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[54] Title of the Invention: AN ANTIMUTAGENIC AGENT

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Specification

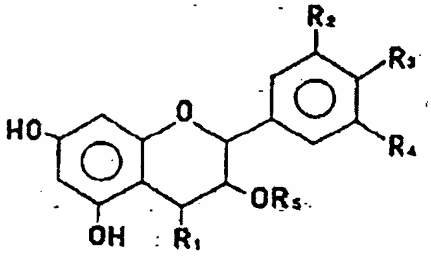
1. Title of the Invention

An Antimutagenic Agent

2. Claims

1. An antimutagenic agent having a proanthocyanidin oligomer as an active ingredient.

2. An antimutagenic agent according to claim 1, wherein the proanthocyanidin oligomer is at least 1 substance selected from among the group consisting of dimers through decamers in which flavan-3-ols or flavan-3,4-diols represented by the following general formula:



(where R_1 is hydrogen or a hydroxyl group, R_2, R_3, and R_4 are hydrogen, a hydroxyl group, or methoxyl group, and R_5 is hydrogen, a galloyl group, or a glycopyranosyl group)

are bonded as structural units.

3. Detailed Description of the Invention

(Industrial Field of Application)

The present invention relates to an antimutagenic agent, in particular, a highly stable antimutagenic agent that deactivates mutagenic substances in food products.

(Prior Art)

Numerous mutagenic substances exist in the living environment that surrounds us. Furthermore, it has been reported by numerous research institutions that the mutagenicity of these substances has a strong correlation with carcinogenicity. This indicates that a reduction in cancer risk in humans can be effected by reducing the mutagenicity of these substances.

Mutanogenic substances existing in foods include the heterocyclic amines in charred

materials, aflatoxins known as mold toxins, and other types of materials, and among these are many which have strong mutagenicity and have been proven to have carcinogenic properties.

In the past, various studies have been performed concerning methods of deactivating these mutagenic substances. One such study has shown the mutagenicity inhibiting action of catechins on benzo[*a*]pyrene.

(Problems that the Invention is to Solve)

The present invention has the object of offering an antimutagenic agent that is able to increase the safety of foods by eliminating or reducing the mutagenicity in food products containing mutagenic substances.

(Means Used to Solve the Problems)

In the present invention, as the result of intensive studies aimed at achieving the aforesaid purpose, it was found that proanthocyanidin oligomers exhibit a strong mutagenicity inhibiting action on mutagenic substances in foods, particularly Trp P-2, which is a representative example of tetracyclic amines in scorched foods, and based on these findings achieved the present invention.

Specifically, the present invention is an antimutagenic agent having proanthocyanidin oligomers as its effective ingredient.

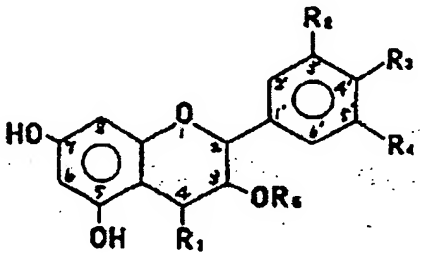
The present invention is explained below in further detail.

First, in the present invention, the term "proanthocyanidin oligomers" refers to condensed type tannins present in various plant matter, specifically, the compound group formed by the bonding of flavan-3-ol or flavan-3,4-diol as structural units by condensation or polymerization and given this name because they produce cyanidin, delfinidin, peralgonidin, etc., when treated with an acid.

Accordingly, proanthocyanidin oligomers such as polymeric procyanidin, prodelfinidin, and properalgonidin, which are dimers, trimers, tetramers, or even decamers or above of the aforesaid structural units, and stearic isomers thereof are all included among said proanthocyanidin oligomers.

Additionally, these proanthocyanidin oligomers, unlike monomeric catechins that are their structural unit, are known to have a mutagenicity inhibiting action with regard to benzo[*a*]pyrene

as described above, and have as their major characteristic the fact that they possess extremely strong mutagenicity inhibiting action. Moreover, it has been discovered that said mutagenicity inhibiting action becomes stronger the more the degree of condensation or degree of polymerization is increased. From this fact, and in terms of solubility, etc., dimers through decamers, in which flavan-3-ols or flavan-3,4-diols represented by the following general formula:



(where R_1 is hydrogen or a hydroxyl group, R_2, R_3, and R_4 are hydrogen, a hydroxyl group, or methoxyl group, and R_5 is hydrogen, a galloyl group, or a glycopyranosyl group) are bonded as structural units (see Japanese Unexamined Patent Application Kokai No. Sho 61-16982) are desirable, and among these, dimers through hexamers are especially desirable from the standpoint of absorptivity in the human body.

Of course, the proanthocyanidin oligomers used in the present invention do not themselves have any mutagenicity [*Food and Chemical Toxicology* (*Fd. Chem. Toxic.*), Vol. 25, p. 135 (1987)]

Said proanthocyanidin oligomers have a mutagenicity inhibiting action on various mutagenic substances, but have especially strong mutagenicity inhibiting efficacy with regard to Trp P-2, one of these mutagenic substances. Specifically, said proanthocyanidin oligomers can be used very effectively in food products containing mutagenic substances such as Trp P-2.

Examples of such food products include heated foods such as broiled fish, hamburgers, Korean-style barbecued meat, etc.

Of course, the present invention can also be used in food products containing other mutagenic substances.

Next, the additive amount of proanthocyanidin oligomers is not subject to any particular restrictions, and normally is in a range of 20 to 20,000 times, preferably 200 to 2000 times, the

molar equivalent of the mutagenic substance. Said additive amount in relation to the food product roughly varies according to the quantity of mutagenic substance in the food product, etc., but generally is in a range of 1×10^{-5} to 10% (W/W), preferably 1×10^{-3} to 1×10^{-1}% (W/W).

Furthermore, the proanthocyanidin oligomers can be used as they are in the form of a powder or liquid, or can be added to an edible substance (such as glucose, dextrin, etc.) and used as an antimutagenic agent, which is added to and blended with the food product. Moreover, said antimutagenic agent can be added to seasonings such as sauces, for example, that are used on heated food products.

There is absolutely no effect on the taste of the foods resulting from the use of said antimutagenic agent.

Here, the proanthocyanidin oligomers used in the present invention are described in detail.

Dimeric through decameric, etc., proanthocyanidin oligomers in which flavan-3-ols or flavan-3,4-diols represented by the aforesaid general formula are bonded as structural units can be obtained by conventional methods, specifically, synthesis processes or extraction from various types of plant matter. Referring to the latter case, for example, various types of plant matter may be subjected to an extraction process using a solvent, and the extract obtained separately refined by means of liquid chromatography, etc. Alternatively, a secondary processed product such as fruit juice, liquor or beer having a plant source material is treated with a selective attractant for proanthocyanidin oligomers, said proanthocyanidin oligomer fraction is condensed, and the condensate is separated and refined by means of a directional flow distribution method, liquid chromatography, etc.

Various types of proanthocyanidin oligomers and their manufacturing methods are cited below.

(1) Dimeric proanthocyanidin B-2 (C_4-C_8 bonded catechin-catechin), C_4-C_8 bonded dimeric procyanidin (C_4-C_8 bonded catechin-afzelechin): using a method of a portion of the present inventors specified in *Agricultural Biological Chemistry* (*Agric. Biol. Chem.*), Vol. 45, pp. 2709-2712 (1981), a 70% aqueous acetone extract of adzuki beans (*Vigna angularis ohwiet ohashi*), is separated and refined by means of liquid chromatography using a column of polyamide C-200 and Sephadex LH-20, and the product is obtained thereby.

(2) Dimeric proanthocyanidin A-2: obtained using the shells of the seed of the horse chestnut (*Aesculus hippocastanum*) by the method specified by D. Jacques, et al., in the *Journal of Chemical Society Perkin I* (*J.C.S. Perkin I*) pp. 2663-2671 (1974).

(3) C_4-C_8 bonded dimeric procyanidin (C_4-C_8 bonded catechin-catechin): obtained using the bark of the pine (Lobololly pine) by the method specified by R.W. Hemingway, et al., in *Phytochemistry*, Vol. 22, pp. 275-281 (1983).

(4) C_6-C_8 bonded dimeric prodelfinidin (C_6-C_8 bonded gallocatechin-catechin): obtained using bark of the oak as the raw material by the method specified by Byung-Zun Ahn, et al., in *Archiv. der Pharmazie und Berichte der Deutschen Pharmazeutischen Gesellschaft* (*Arch. Pharmaz.*), pp. 666-673 (1970).

(5) Procyanidin B-1-gallate (C_4-C_8 bonded catechin gallate-catechin), procyanidin B-1 digallate (C_4-C_8 bonded catechin gallate-catechin gallate): obtained using the roots of knotgrass [Japanese: *michiyanagi*] (*Polygonum multiflorum*) by the method of Nonaka, et al., in *Phytochemistry*, Vol. 21, pp. 429-432 (1982).

(6) Dimeric prodelfinidin B-2 digallate (C_4-C_8 bonded gallocatechin gallate-gallocatechin gallate): obtained using the bark of the myrica [Japanese: *yamamomo*] (*Myracarubra*) by the method specified by Nonaka, et al., in *Phytochemistry*, Vol. 22, pp. 237-241 (1983).

(7) C_4-C_8 bonded dimeric peralgonidin (C_4-C_8 bonded afzelechin-catechin), C_4-C_8 bonded trimeric prodelfinidin (C_4-C_8 bonded gallocatechin-gallocatechin-catechin): obtained using as raw materials barley and barley sprouts in accordance with the method specified by I. McMurrough, et al., in the *Journal of Science of Food Agriculture* (*J. Sci. Food Agric.*), Vol. 34, pp. 62-72 (1983).

(8) Dimeric procyanidin B-4 rhamnoside: obtained by the method specified in Japanese Unexamined Patent Publication Kokai Sho 59-59638, using the *mehirugi* [*Kandelia Rheedii*].

(9) C_4-C_8 bonded dimeric properalgonidin [C_4-C_8 bonded afzelechin-gallocatechin (4'-O-methyl)]: obtained using the skin of Ouratea roots in accordance with the method specified by F.D. Monache, et al., in *Annali de Chimica* [*Ann. Chim.* (Rome)], Vol. 57, pp. 1364-1371 (1967).

(10) C_4-C_8 bonded tetrameric procyanidin (C_4-C_8 bonded catechin-catechin-catechin-catechin): obtained in accordance with the method specified by A.G.H. Lea in the *Journal of*

Science of Food Agriculture (*J. Sci. Food Agric.*), Vol. 29, pp. 471-477 (1978), by separation and purification of a condensate of proanthocyanidin obtained by treating fermented apple cider with Sephadex LH-20, by means of a directional flow distribution method using ethyl acetate and water and liquid chromatography using a column of LH-20.

(11) Dimeric procyanidin B-3 (C_4-C_8 bonded catechin-catechin), dimeric procyanidin B-4 (C_4-C_8 bonded catechin-catechin): obtained by means of a synthesis process using dihydroquercetin and catechin or epicatechin in accordance with the method specified by G. Fonknechten, et al., in *Journal of Institute Brewing* (*J. Inst. Brew.*), Vol. 89, pp. 224-231 (1983). Also obtained by the synthesis method specified by R. Eastmond in the *Journal of Institute Brewing* (*J. Inst. Brew.*), Vol. 80, p. 188 (1974).

Working examples are described below.

In the working examples, as the method of measuring mutagenicity and the mutagenicity inhibiting action, a preincubation method [modified version of the Ames method established as a carcinogenicity screening process, see Suginama, Nagao: *Chemical Mutagens*, Vol. 6, p. 41 (1981)], and *Salmonella typhymurium*, strain TA98 (hereinafter referred to as strain TA98), which has hystidine-demanding properties, was used as the bacterial strain. The following formula was also used:

$$\text{Mutagenicity inhibition ratio (Y\%)} = 1\text{-}[(A\text{-}C)/(B\text{-}C)] \times 100$$

A: colony number/plate on antimutagenic agent-added plate

B: colony number/plate on antimutagenic agent non-added plate [corresponding in the table to the row added amount 0 (not added)]

C: natural reversion colony number/plate

(A-C) and (B-C): reverse mutation colony number/plate

Working Example

I. Mutagenicity inhibiting action of catechin and proanthocyanidin oligomer on Trp P-2 (3-amino-1-methyl-5H-pyrido[4,3-b],indol)

Catechin (commercially sold product) in the amount shown in Table 1, 50 $\mu\ell$ each of samples of dimeric procyanidin B-4 (referred to as dimeric PC in the table), which is one proanthocyanidin oligomer, and tetrameric procyanidin (referred to as tetrameric PC in the table) having C_4-C_8 bonds (obtained by the following method of production), together with 500 $\mu\ell$ of S-9 mix and 100 $\mu\ell$ of a preculture solution of strain TA98, were added to 50 $\mu\ell$ of an aqueous solution containing 0.066% (W/V) of the mutagenic substance Trp P-2. This mixed liquid was immediately incubated for 20 min at 37°C, next 2 mℓ of agar containing 0.5 mM hystidine and 0.5 mM biotin was added thereto, and the blend was spread on a small amount of glucose agar medium. Then, after still culturing for 48 hrs at 37°C, the number of colonies on the plate were counted. The results are shown in Table 1.

Method of producing proanthocyanidin oligomers:

(1) Dimeric proanthocyanidin B-3 (C_4-C_8 bonded catechin-catechin):

In accordance with the method of R. Eastmond described above, a synthesis reaction was performed using 5 g (±) dihydroquercetin, 5 g (+)-catechin, and sodium borohydride as source materials. After the reaction was completed, the pH was adjusted to 5.0 with acetic acid, and extraction was performed with ethylacetate. The liquid extract obtained was vacuum-distilled, this was fractionated using Sephadex LH (diameter 2.5 × 67 cm) as a carrier in column chromatography using ethanol as a developing solvent. Coarse procyanidin B-3 was obtained by separating a fraction of the elution liquid in the amount of 900 to 1300 mℓ. This fraction was further refined using reverse-phase type silica gel high-speed liquid chromatography (column μ Bondapak C_{18} [19 × 150 mm]; developing solvent 7.5% methanol; detection $OD_{280\ nm}$) and 507 mg of the target substance was obtained.

(2) C_4-C_8 bonded tetrameric procyanidin (C_4-C_8 bonded catechin-catechin-catechin-catechin):

2 ℓ of water was added to 1 kg of white grape seeds obtained by separating white grape (variety: Chardonnay) pressed lees with a sieve, and extraction was performed for 2 hrs at 85°C. The liquid extract obtained by solid-liquid separation was filtered with a polyester based resin SP207 (manufactured by Mitsubishi Chemical Corp.) column (diameter 1.8 × 50 cm), and polyphenol was adsorbed in the column. This column was washed with 1.5 ℓ of water and 1.5 ℓ

of 15% ethanol, and then eluted with 1.5 ℓ of 30% ethanol. The eluted liquid was vacuum-condensed, then freeze-dried, and 20.4 g of coarse proanthocyanidin oligomer (proanthocyanidin oligomer dimer-hexamer content 51%) was obtained. Using 2 g of this powder, Sephadex LH-20 (diameter 2.5 × 66 cm), using 2600 mℓ ethanol and 2600 ℓ methanol as development solvents (2 stage elution), and column chromatography was performed with detection in the ultraviolet range ($OD_{240\ nm}$). 3200 to 3400 mℓ of the fraction were extracted from this elution liquid, and 150 mg of target substance obtained in accordance with the aforesaid method of A.G.H. Lee.

S-9 mix: A blend of 10 μℓ of the following S-9 and 490 μℓ of cofactor mix.

S-9 [manufactured by Kikkoman Corp.]: The centrifugally processed (9000 × g, 10 min) supernatant of rat liver homogenates derived from drug metabolizing enzymes when phenobarbitol and 5,6-benzoflavone were administered in the abdominal cavity of 7 week old male SD rats as an inducer.

Cofactor mix [commercial product name, manufactured by Oriental Yeast Co., Ltd.]: nicotinamide dinucleotide (NADH), nicotinamide dinucleotide phosphoric acid (NADH), glucose-6-phosphoric acid (G6P) enzymes, etc., and magnesium ions (Mg^{3+}), etc., were added.

Table 1

Additive amount (mg/plate)	Catechin	Dimeric PC	Tetrameric PC
	A (Y%)	A (Y%)	A (Y%)
0	4000 (0)	4000 (0)	4000 (0)
125	4000 (0)	3200 (20)	274 (94)
250	2400 (40)	2000 (50)	109 (98)
500	1508 (63)	1205 (70)	106 (98)

*Natural reversion colony number/plate: 23

From Table 1 it can be seen that the mutagenicity in inhibition ratio (antimutagenicity) of

proanthocyanidin oligomers with regard to Trp P-2 in the presence of the rat liver homogenate is clearly manifested and is much higher than that of catechin.

II. Mutagenicity inhibiting activity of catechin and proanthocyanidin oligomers with regard to activated Trp P-2.

The aforesaid I in additive amounts shown in Table 2, 50 $\mu\ell$ of samples of catechin and proanthocyanidin oligomer, 500 $\mu\ell$ of 100 mM sodium phosphate buffer solution, and 100 $\mu\ell$ of strain TA98 preculture solution were added to 50 $\mu\ell$ of activated Trp P-2 prepared in accordance with the following method of Hayatsu, et al. The blended liquid was immediately incubated for 20 min at 37°C, then 2 mℓ of soft agar solution containing 0.5 mM histidine and 0.5 mM biotin were added thereto, and spread on a small amount of glucose agar medium. After still culturing for 48 hrs at 37°C, the number of colonies on the plate were counted. The results are shown in Table 2.

Preparation of activated Trp P-2 [method of Hayatsu, et al.: *Biochemical and Biophysical Research Communications* (*Biochem. Biophys. Res. Commun.*), Vol. 92, pp. 662-668 (1980)]: 500 $\mu\ell$ of the aforesaid S-9 mix was added to 50 mℓ of Trp P-2, and this liquid mixture was immediately incubated for 20 min at 37°C. Next, an equal amount of acetone was added thereto, ice cooling was performed, and after 15 min centrifugal treatment was performed at 3000 rpm for 10 min, the supernatant obtained was vacuum dried, 50 $\mu\ell$ of sterilized water was added, and activated Trp P-2 was prepared.

Table 2

Additive amount (mg/plate)	Catechin	Dimeric PC	Tetrameric PC
	A (Y%)	A (Y%)	A (Y%)
0	1373 (0)	1373 (0)	1373 (0)
125	610 (57)	305 (79)	204 (87)
250	377 (75)	77 (96)	69 (97)
500	53 (98)	49 (98)	46 (98)

*Natural reversion colony number/plate: 22

From Table 2 it can be seen that the mutagenicity inhibition ratio of the proanthocyanidin oligomer with regard to activated Trp P-2 was clearly manifested and was dramatically higher than that of catechin.

From the foregoing it can be concluded that proanthocyanidin oligomers are superior as antimutagenic agents, and also have a clearly stronger action than catechin, which is already known to have antimutagenic action against benzo[*a*]pyrene.

Patent Applicant: Kikkoman Corp.